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Role of Zn^{2+} in restoration of nonprotein thiol content in the cells under chemical stress induced by triclocarban

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Abstract: We have proposed that intracellular Zn^{2+} release during oxidative stress is a trigger to restore cellular thiol content that is decreased by oxidative stress. Recently, we found that the incubation with triclocarban for 1 h decreased cellular thiol content and increased intracellular Zn^{2+} concentration. It was reminiscent of the possibility that the increase in intracellular Zn^{2+} concentration by triclocarban could become a trigger to restore the cellular content of nonprotein thiols. To test the possibility, we cytometrically examined the effects of prolonged incubation (3 h) with triclocarban on the cellular content of nonprotein thiols, presumably glutathione, by the use of 5-chloromethylfluorescein (5-CMF) diacetate, a fluorescent indicator of cellular nonprotein thiols, in rat thymocytes. The intensity of 5-CMF fluorescence after the 3 h incubation with 300 nM triclocarban was significantly higher than that after the 1 h incubation. In the presence of a chelator of intracellular Zn^{2+}, such a significant difference was not observed. The results suggest that the increase in intracellular Zn^{2+} concentration by triclocarban is one of triggers to restore cellular content that is decreased by triclocarban.

Key words: *Intracellular Zn^{2+}; Chemical stress; Intracellular thiol; Triclocarban*

1. Introduction

Although the ability of zinc to retard oxidative process has been recognized for many years (Powell, 2000; Prasad, 2008), zinc itself has been reported to induce oxidative stress (Kim et al., 1999; Capasso et al., 2005; Bishop et al., 2007; Matsui et al., 2009). In our previous study (Kinazaki et al., 2011), the effects of N-ethylmaleimide and $ZnCl_2$ on cellular thiol content and intracellular Zn^{2+} concentration were examined in rat thymocytes. The treatment of cells with N-ethylmaleimide decreased cellular content of nonprotein thiol and increased intracellular Zn^{2+} concentration. Micromolar $ZnCl_2$ increased both intracellular Zn^{2+} concentration and cellular content of nonprotein thiol. It was hypothesized that the increase in intracellular Zn^{2+} concentration during oxidative stress might be a trigger to restore cellular thiol content that was decreased by oxidative stress.

Whereas the incubation with tri-n-butyltin for 1 h greatly decreased the cellular content of nonprotein thiol, the cellular content restored during the prolonged incubation with tri-n-butyltin (Okada et al., 2000). Tri-n-butyltin also increased intracellular Zn^{2+} concentration (Oyama et al., 2009). It was reminiscent of the possibility that tri-n-butyltin-induced increase in intracellular Zn^{2+} concentration might elicit the restoration of cellular content of nonprotein thiol.

Taken together, it may be a common feature that the chemicals decreasing cellular content of nonprotein thiol increase intracellular Zn^{2+} concentration, leading to restore the cellular content of nonprotein thiol. In our recent studies (Morita et al., In accepted), nanomolar concentrations of triclocarban (3,4,4′-trichlorocarbanilide, CAS 101-20-2) that was detected in human blood after showering with soap containing triclocarban (Schebb et al., 2011, 2012) decreased cellular content of nonprotein thiol and increased intracellular Zn^{2+} concentration in rat thymocytes. Therefore, it may be also the case. To see if the prolonged incubation with nanomolar triclocarban restores cellular content of nonprotein thiol in the Zn^{2+}-dependent manner, the effects of triclocarban on intracellular Zn^{2+} concentration and cellular content of nonprotein thiol of rat thymocytes were examined in absence and presence of Zn^{2+}-chelators by the uses of FluoZin-3 and 5-chloromethylfluorescein fluorescence with a flow cytometer. Triclocarban is commonly used as an antimicrobial agent in personal care products. Triclocarban has attracted some public attention

because of reports that it acts as an endocrine disruptor (Chen et al., 2008; Giudice and Young, 2010). In contrast, there is very limited information concerning the cytotoxic actions of triclocarban in mammalian cells, although nanomolar concentrations of triclocarban were detected in human blood following the use of soap containing triclocarban (Schebb et al., 2012). This study may also give some insights into the cytotoxicity of triclocarban.

2. Materials and methods

2.1. Chemicals

Triclocarban was purchased from Wako Pure Chemicals (Osaka, Japan). The chelator for Zn^{2+}, N,N,N′,N′-tetrakis[2-pyridylmethyl]ethylenediamine (TPEN), was obtained from Dojin Chemical Laboratory (Kumamoto, Japan). The pH buffer was 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Nacalai Tesque, Kyoto, Japan). 5-Chloromethylfluorescein diacetate (5-CMF-DA) and propidium iodide were purchased from Molecular Probes Inc. (Eugene, Oregon, USA). Other chemicals were purchased from Wako Pure Chemicals.

2.2. Animals and cell preparation

The study was approved by the Committee for Animal Experiments of the University of Tokushima (Registration No. 05279). The cell suspension was prepared in a similar manner to that previously reported (Chikahisa et al., 1996). In brief, thymus glands dissected from ether-anesthetized rats were sliced at a thickness of 400–500 μm with a razor blade under cold conditions (3–4°C). The slices were triturated by gentle shaking in chilled Tyrode's solution to dissociate the thymocytes. Thereafter, the Tyrode's solution containing the cells was passed through a mesh (size: 10 μm) to prepare the cell suspension. The beaker containing the cell suspension was incubated in a water bath at 36–37°C for 1 h before the experiment. Although Tyrode's solution did not contain $ZnCl_2$, the cell suspension generally contained 200–230 nM zinc derived from the cell preparation (Sakanashi et al., 2009).

2.3. Fluorescence measurements of cellular and membrane parameters

The methods for the measurements of the cellular and membrane parameters by using a flow cytometer equipped with an argon laser (CytoACE-150, JASCO, Tokyo, Japan) and fluorescent probes were similar to those previously described (Chikahisa et al., 1996; Matsui et al., 2008). The fluorescence was analyzed using JASCO software (Version 3XX, JASCO). There was no fluorescence from the reagents used in the study, except for the fluorescent probes, under our experimental conditions. To assess cell lethality, propidium iodide was added to the cell suspension to achieve a final concentration of 5 μM. Since propidium stains dead cells, the measurement of propidium fluorescence from cells provided an estimation of cell lethality. The fluorescence was measured with a flow cytometer 2 min after the application of propidium iodide. The excitation wavelength used for propidium was 488 nm and emission was detected at 600 ± 20 nm. 5-CMF-DA was used to monitor changes in the cellular content of non-protein thiol, presumably glutathione (Chikahisa et al., 1996). The cells were incubated with 1 μM 5-CMF-DA for 30 min before any fluorescence measurements. 5-CMF fluorescence was measured in the cells that were not stained with 5 μM propidium iodide. The excitation wavelength used for 5-CMF was 488 nm and emission was detected at 530 ± 15 nm.

2.4. Statistics

Values were expressed as the mean ± standard deviation of 4 experiments. Statistical analysis was performed with Tukey's multivariate analysis. A P value of < 0.05 was considered significant.

3. Results and Discussion

3.1. Effects of short and prolonged exposures to triclocarban on viability and cellular content of nonprotein thiols

The incubation with triclocarban at concentrations ranging from 10 nM to 1 μM for 1 h did not affect the viability of rat thymocytes. As shown in Fig. 1, the incubation of triclocarban for 1 h significantly attenuated 5-CMF fluorescence when the concentration of triclocarban was 300 nM or more (up to 1 μM). These results suggest that triclocarban at 300 nM or more significantly decreased cellular content of nonprotein thiol, presumably glutathione (Chikahisa et al., 1996). On the contrary, the incubation of 300 nM triclocarban for 3 h augmented 5-CMF fluorescence whereas the intensity of 5-CMF fluorescence monitored from the cells treated with 1 μM triclocarban for 3 h was significantly lower than control intensity (Fig. 1). Thus, the cells were supposed to recover the initial lose of cellular glutathione in the continued presence of 300 nM triclocarban.

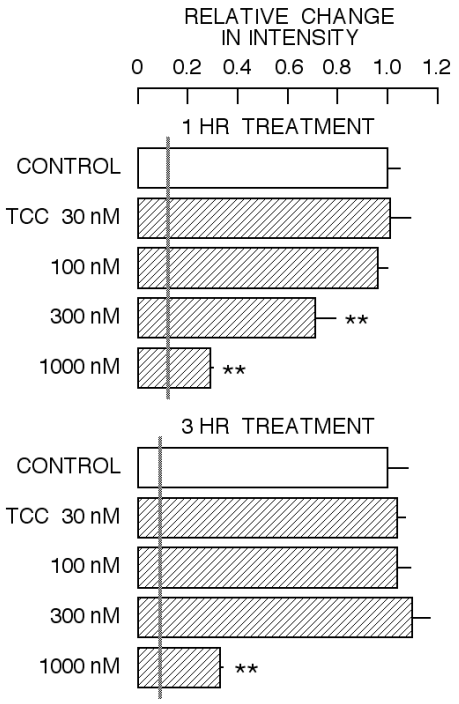

Figure 1. *Relative changes in 5-CMF fluorescence intensity by 1 h and 3 h incubation with triclocarban (TCC). Each column and bar respectively indicate mean and S.D. of four experiments. Asterisks (**) show significant difference (P < 0.01) between control group and TCC-treated group.*

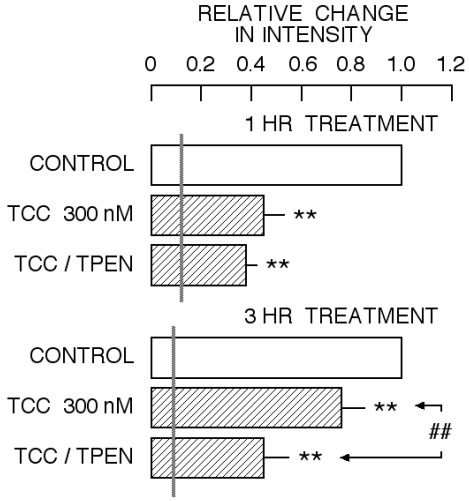

Figure 2. *Effect of TPEN on TCC-induced change in 5-CMF fluorescence. Each column and bar respectively indicate mean and S.D. of four experiments. Asterisks (**) show significant difference (P < 0.01) between control group and TCC-treated group. Symbol (##) shows significant difference (P < 0.01) between arrowed pair.*

3.2. Effect of TPEN on restoration of cellular content of nonprotein thiols

The incubation with triclocarban at concentrations of 100 nM or more significantly augmented FluoZin-3 fluorescence in a concentration-dependent manner (Morita et al., In accepted), indicating the increase in intracellular Zn^{2+} concentration. The elevation of intracellular Zn^{2+} concentration increases cellular thiol content (Kinazaki et al., 2011). Therefore, the effect of a chelator for intracellular Zn^{2+} (TPEN) on the cells treated with 300 nM triclocarban was examined to reveal the contribution of intracellular Zn^{2+} to the restoration of cellular content of nonprotein thiols. As shown in Fig. 2, the incubation with 300 nM triclocarban for 1 h significantly decreased the 5-CMF fluorescence. There was no significant difference in 5-CMF fluorescence between the groups of cells treated with triclocarban for 1 h. However, after 3 h incubation with triclocarban, the intensity of 5-CMF fluorescence of cells treated with triclocarban in the presence of TPEN was significantly lower than that in the absence of TPEN.

3.3. Effect of low temperature on restoration of cellular content of nonprotein thiols

In the case of 3 h treatment with triclocarban, cooling to 1–4 ºC was made during 0.5–2.5 h after the start of triclocarban application.

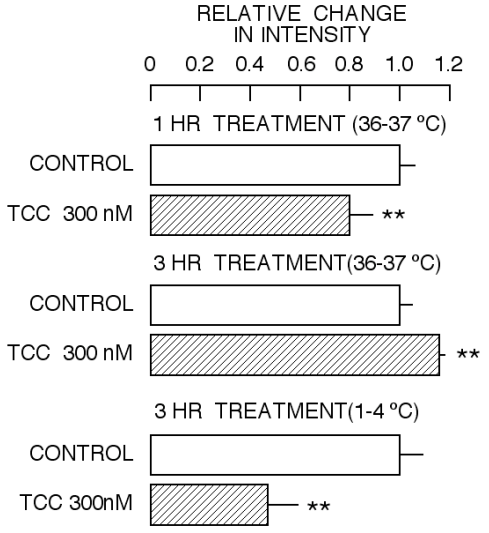

Figure 3. *Effect of low temperature on TCC-induced change in 5-CMF fluorescence. Each column and bar respectively indicate mean and S.D. of four experiments. Asterisks (**) show significant difference (P < 0.01) between control group and TCC-treated group.*

As shown in Fig. 3, the restoration of cellular thiol content was occurred under normal temperature after the 3 h incubation with 300 nM triclocarban. However, no restoration was observed in the case of the cells suffering from cooling (1–4 ºC). Thus, the cooling may block the Zn^{2+}-induced increase in *de novo* synthesis of nonprotein thiols (Ha et al., 2006; Cortese et al., 2008).

One may argue that the level of 5-CMF fluorescence in the presence of 300 nM triclocarban varied from experiment to experiment in this study although the tendency of triclocarban-induced change was same. The variation was probably due to the difference in cell ability to restore cellular content of nonprotein thiol, responding to the increase in intracellular Zn^{2+} concentration by triclocarban, and due to the reciprocal actions of triclocarban that triclocarban decreased cellular content of nonprotein thiols and the triclocarban-induced elevation of intracellular Zn^{2+} concentration increased cellular content of nonprotein thiols.

From the results, it is suggested that the increase in intracellular Zn^{2+} concentration by triclocarban becomes a trigger to restore the cellular content of nonprotein thiols that are decreased by triclocarban.

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